COMPARISON OF BIOMARKERS FOR EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE: COTININE AND HAEMOGLOBIN ADDUCTS FROM AROMATIC AMINES AND TOBACCO-SPECIFIC NITROSAMINES IN PREGNANT SMOKING AND NONSMOKING WOMEN

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INTRODUCTION

Exposure to environmental tobacco smoke (ETS) has been suggested as a possible risk factor for lung cancer in several, but not all, epidemiological studies.[1,2] A recent meta-analysis performed by the US Environmental Protection Agency[3] estimated a significant risk (RR = 1.3; $p<0.05$) for ETS exposure and lung cancer in nonsmokers. Such an assessment is only valid if miss-classification of smokers as nonsmokers and other possible confounding factors have been correctly taken into consideration. The validity of determining cotinine in urine and plasma as a biomarker of ETS exposure over the last few days has recently been demonstrated in a large-scale study carried out in Southern Germany.[4] Assessment of long-term ETS exposure remains difficult since no reliable biomarkers are available. Adducts from tobacco-specific nitrosamines (TSNA) and aromatic amines such as 4-aminobiphenyl (4-ABP) have been proposed as biomarkers in both smokers and nonsmokers.[5,6] The long half-life of haemoglobin adducts make them especially suitable as surrogate markers of exposure occurring over an extended period of several months.

So far only a few studies have investigated the influence of involuntary exposure to ETS upon haemoglobin adduct levels.[7-9] Levels of 4-ABP were marginally higher in nonsmokers with detectable cotinine in their plasma ($p=0.05$). A more significant difference was found for 3-ABP ($p= 0.027$).[7] More recently, a significant increase of 4-ABP adducts was reported in nonsmoking pregnant women with increasing ETS exposure ($p = 0.009$).[8] Exposure to ETS was quantified for 1 week by a personal diary and by air sampling of nicotine. We have investigated plasma and urinary cotinine and haemoglobin adducts from 9 aromatic amines and the TSNA-derived adduct, 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), in self-reported smoking and nonsmoking women from Homburg, Germany.

SUBJECTS

One hundred and five pregnant women attending the University Hospital of Homburg were recruited for this study in 1993. All subjects were interviewed using a questionnaire containing 180 questions on general health, life style, occupation, smoking habits and ETS exposure. Twenty-seven women claimed to be active smokers, whereas 78 women declared themselves to be nonsmokers. The nonsmokers were subdivided into four groups according to their self-reported passive smoke exposure (Fig. 1). There were no significant differences in age distribution between nonsmokers (29.6±5.2 years) and smokers (28.7±6.2 years; $p > 0.10$). Fifty-five and 52% of the nonsmokers and smokers, respectively, were white collar workers and about one third in both groups were not practising a profession ($p>0.05$). There was a tendency to a lower educational level in smokers with 68% having no secondary education as compared to 47.4% of nonsmokers. A higher percentage of smokers (33.2%) than nonsmokers (14.1%) were unmarried. However, these differences did not reach statistical significance ($p>0.05$).

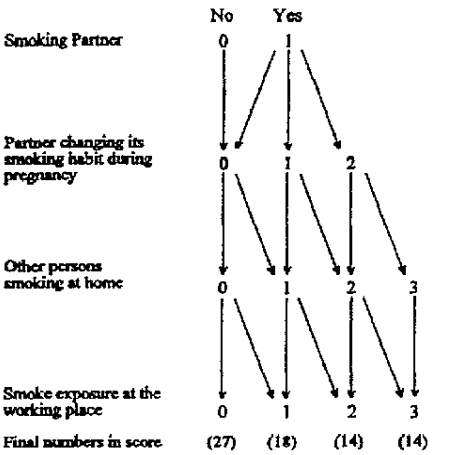

Figure 1. Passive smoke score in pregnant nonsmoking women form Homburg

METHODS

During delivery a spot urine and 20 ml of blood were collected. Red blood cells were separated by centrifugation, divided in two portions and washed three times with twice the volume of saline. A 2 ml aliquot of plasma was taken for analysis of cotinine. All samples were frozen at -20°C until analysis.

The analytical procedure used in this study was essentially the same as reported previously[10]. Briefly, cotinine in plasma and urine was determined by a radioimmunological method. The haemoglobin concentration was determined by the Drabkin method (Sigma kit No. 525A). To dialysed blood samples 40 pg D_9-4-ABP, 80 pg D_5-aniline and 20 pg D_5-HPB were added as internal standards. After mild alkaline hydrolysis the solutions were applied to C18 Bond Elut extractions tubes. The retained analytes were eluted with chloroform and divided in two fractions for derivatisation of aromatic amines and HPB with pentafluoropropionic anhydride and pentafluorobenzoyl chloride, respectively. GC-MS analysis was performed by negative chemical ionization with methane in the SIM mode using a QMD 1000 GC-MS equipped with a DB5.ms capillary column (Fisons, Mainz, Germany). Blank samples consisting of water were run each day. All samples were analysed in duplicate and the mean values calculated.

RESULTS

Five of 78 self-reported nonsmoking women (6.7%) had plasma cotinine levels indicative of current smoking (36, 38, 90, 93, and 150 ng/ml). They were omitted from statistical evaluation (Tables 1 and 2) but are shown in Figure 3 as miss-classified nonsmokers. Likewise, 4 of 27 self-reported smokers had plasma cotinine levels (<0.5, <0.5, 1.0, and 2.0 ng/ml) indicating that they had not smoked over the last 2-3 days . Because of the long half-life of haemoglobin

adducts, these subjects were not omitted from statistical evaluation and are shown in Figure 3 as smokers with a plasma cotinine level <15 ng/ml.

The mean results for all haemoglobin adducts and for urinary cotinine are summarized in Table 1. Compared to nonsmoking pregnant women, smoking pregnant women had significantly higher levels of urinary cotinine (134-fold), HPB (2-fold) and five of nine aromatic amines, 3-ABP (2.2-fold) and 4-ABP (2.7-fold), *o*- and *p*-toluidine (1.2- and 1.6-fold), and 2,4-dimethylaniline (1.4- fold). However, none of these haemoglobin adducts can be regarded as being specific for smoking because of the considerable overlapping ranges (Fig. 3). For 2-ethylaniline (1.3-fold) the difference did not reach statistical significance. Aniline, *m*-toluidine and *o*-anisidine were detected in all blood samples with similar levels in smoking and nonsmoking women. Plasma cotinine levels in smoking pregnant women were 103±16 ng/ml. In nonsmokers, cotinine was detectable (>0.5 ng/ml) in only 15 of 73 plasma samples (range 0.5-4.0 ng/ml).

Table 1. Haemoglobin adducts (fmol/g haemoglobin) and cotinine in urine (ng/mg creatinine) in smoking and nonsmoking pregnant women from Homburg

	Aniline	*o*-Toluidine	*m*-Toluidine	*p*-Toluidine	2-Ethylaniline	2,4-Dimethylaniline	*o*-Anisidine	3-ABP	4-ABP	HPB	Urinary Cotinine
	Nonsmoking pregnant women (n = 73)										
Mean	13315	2210	6590	1841	158	154	789	8.0	60.1	26.7	18.9
S.E.	1041	603	513	117	14	13	70	0.7	3.9	4.1	3.6
Min.	2083	224	1545	355	33	25	81	0.0	23.6	0.0	1.7
Max.	56540	43210	23808	5366	520	479	2469	35.5	212.7	229.0	179.9
Median	11544	1330	5054	1652	107	107	536	5.9	53.2	18.0	6.4
	Smoking pregnant women (n = 27)										
Mean	12784	2694	6271	2940	206	210	651	17.7	164.6	54.7	2525
S.E.	1313	233	617	296	27	24	96	3.3	20.2	8.9	389
Min.	3270	560	1657	943	37	41	97	0.0	35.5	0.0	5
Max.	31143	5128	12972	8451	660	503	2079	82.7	384.1	225.0	5284
Median	11061	2371	8385	2646	173	190	503	14.8	150.7	42.0	2647
	Statistical evaluation of smokers vs. nonsmokers (Mann-Whitney)										
p </=	0.938	<0.0001	0.938	<0.001	0.057	0.018	0.374	<0.001	<0.0001	<0.001	<0.0001

Pearson analysis gave no significant positive correlations between urinary cotinine and haemoglobin adducts in nonsmokers (Table 2). However, for smoking pregnant women weak positive correlations were observed for 3-ABP (p=0.0572), 4-ABP (p=0.0691) and HPB (p=0.0574). Pearson analysis with plasma cotinine was possible only in smokers and showed significant positive correlations with aniline, *o*- and *p*-toluidine (p<0.01), 3- and 4-ABP (p<0.05) but not HPB (p>0.1). Adducts from monocyclic aromatic amines were highly correlated with each other in nonsmoking but less so in smoking pregnant women. In contrast, the correlation between 3- and 4-ABP appears stronger in smokers (p=0.0032) than nonsmokers (p=0.0431). Correlations between adducts of monocyclic and bicyclic amines were rather weak and usually not significant. HPB adducts correlated only weakly with 4-ABP in smoking (p=0.0501) but strongly in nonsmoking women (p<0.001).

	Urinary Cotinine
0.067	-0.061
0.010	-0.071
0.087	-0.216*
0.092	-0.042
[illegible]	-0.102
[illegible]	-0.092
[illegible]	-0.087
[illegible]	-0.010
[illegible]	-0.067

* Plasma $p<0.01$.

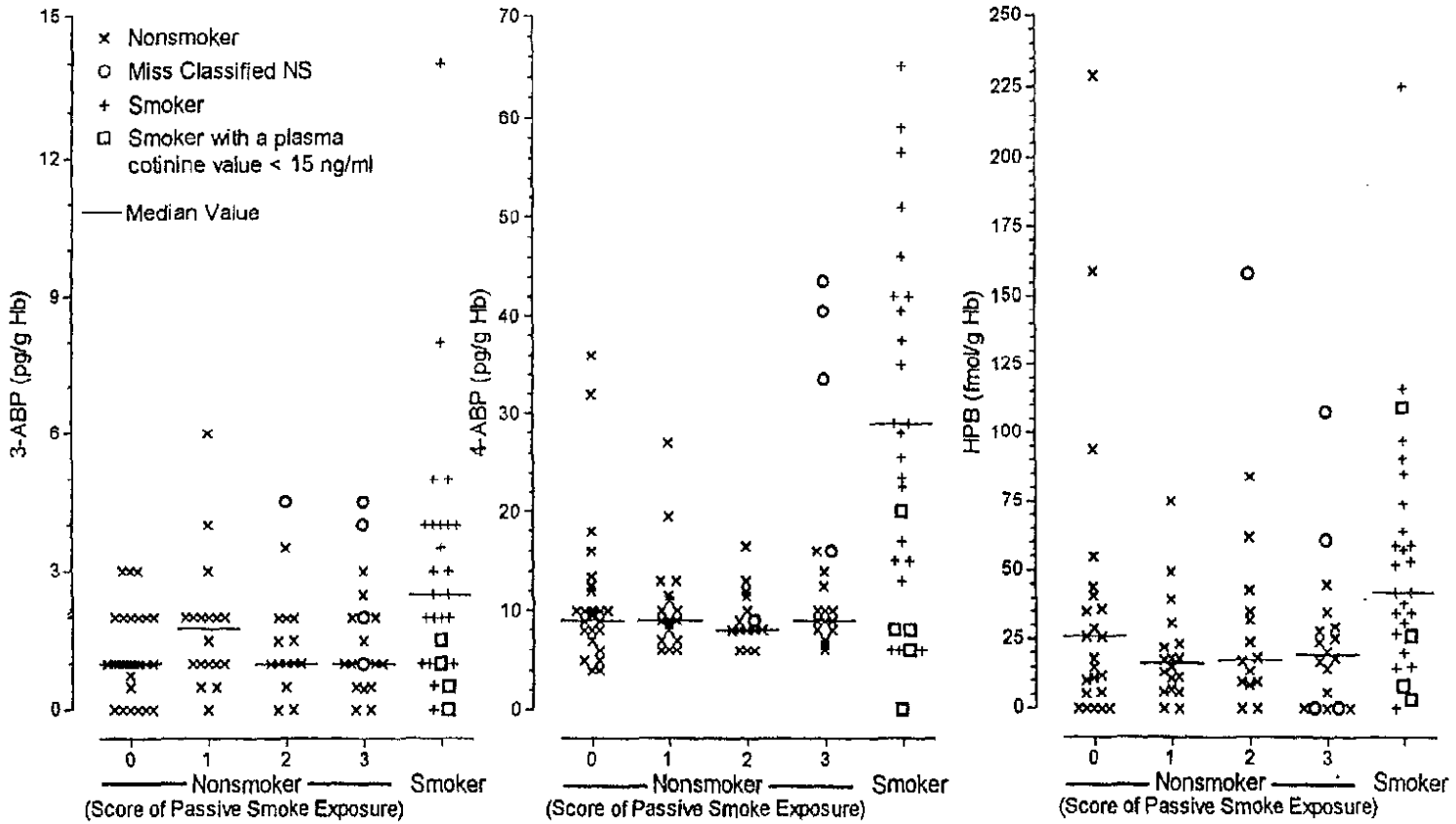

Figure 3. Haemoglobin adducts in self-reported smoking and nonsmoking pregnant women from Homburg; the nonsmokers are subdivided according to their self-reported passive smoke exposure as defined in Figure 1.

Table 2. Pearson correlation coefficients between haemoglobin adducts and cotinine in plasma and urine from pregnant smoking and nonsmoking women from Homburg; $^{*}p<0.05$, $^{**}p<0.01$.

Nonsmoking pregnant women (n = 73; upper right triangle)

	Aniline	*o*-Toluidine	*m*-Toluidine	*p*-Toluidine	2-Ethylaniline	2,4-DMA	*o*-Anisidine	3-ABP	4-ABP	HPB	Urinary Cotinine
Aniline		0.006	0.512**	0.438**	0.489**	0.620**	0.576**	0.287**	0.012	0.043	-0.061
o-Toluidine	0.612**		0.018	-0.057	-0.005	0.002	0.003	-0.034	0.005	0.058	-0.071
m-Toluidine	0.265	0.358*		0.536**	0.633**	0.735**	0.593**	0.207*	0.229*	0.304**	-0.216*
p-Toluidine	0.706**	0.709**	0.230		0.585**	0.594**	0.579**	0.276**	0.296**	0.305**	-0.042
2-Ethylaniline	0.143	0.252	0.553**	0.236		0.909**	0.664**	0.246*	0.137	0.417**	-0.102
2,4-DMA	0.177	0.249	0.600**	0.259	0.915**		0.673**	0.199*	0.025	0.253*	-0.159
o-Anisidine	0.193	0.065	0.434*	0.264	0.375*	0.333*		0.228*	0.024	0.079	-0.092
3-ABP	0.052	0.010	-0.074	0.026	0.048	0.065	-0.243		0.202*	0.156	0.087
4-ABP	-0.047	-0.005	-0.078	-0.036	0.243	0.140	-0.201	0.512**		0.548**	0.010
HPB	-0.002	0.063	-0.237	-0.085	-0.102	-0.235	-0.207	0.010	0.323		-0.067
Urinary Cotinine	0.318	0.255	0.082	0.249	0.139	0.183	-0.122	0.317	0.299	0.317	
Plasma Cotinine	0.501**	0.484**	0.151	0.470**	0.059	0.121	-0.195	0.434*	0.359*	-0.019	

Smoking pregnant women (n = 27; lower left triangle)

Subdividing the nonsmoking women in two classes according to their cotinine levels (ng/mg creatinine) in urine (class I: <10, n = 41 and class II: ≥10, n = 32, respectively) resulted in a 7-fold difference in the mean concentration of cotinine (4.7±0.3 vs 34.0±6.5), however, no differences were found between haemoglobin adduct levels of 3- and 4-ABP as well as HPB (Fig 2). There was a tendency for lower adduct levels in class II as compared to class I for the monocyclic amines aniline (14501±1618 vs 11795±1130), *o*-toluidine (2902±1100 vs 1389±120), *m*-toluidine (7328 ±702 vs 5645±725), *p*-toluidine (1997±180 vs 1642±132), 2-ethylaniline (182±20 vs 127±15), 2,4-dimethylaniline (175±19 vs 126±16) and *o*-anisidine (901±105 vs 646±81).

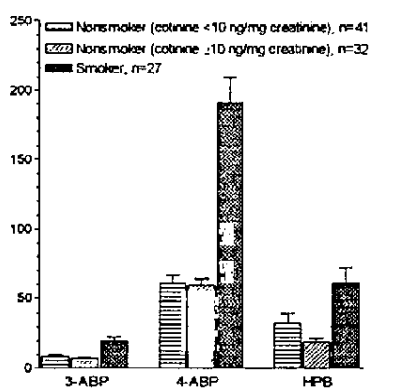

Figure 2. Haemoglobin adducts in self-reported smoking and nonsmoking pregnant women; the nonsmokers are subdivided according to their urinary cotinine levels. Each bar represent the mean ± S.E.

Subdividing the nonsmoking women into four groups according to their self-reported score for ETS exposure showed no influence upon haemoglobin adducts of bicyclic aromatic amines and the TSNA-derived adduct HPB (Fig. 3). For better comparison with literature data the results for 3- and 4-ABP are shown in pg rather than fmol per g haemoglobin. No influence of ETS exposure was found for monocyclic amine adducts (data not shown).

DISCUSSION

The results of this study clearly indicate that there is no influence of ETS exposure on haemoglobin adduct levels of aromatic amines and tobacco-specific nitrosamines in pregnant nonsmoking women from Homburg, Germany. This is in contrast to the results reported by Hammond et al.[8] for 4-ABP in pregnant nonsmoking women from Worcester, MS, USA and by Maclure et al.[7] for both 3- and 4-ABP in adults from either Boston, MS, USA or from Italy. Interestingly, the 4-ABP haemoglobin levels in nonsmoking pregnant women (10.2± 0.7) were significantly lower than that reported by Hammond et al.[8] (22±1.3 pg/g), for adults resident in Munich, Germany[10] (28.8±2.7 pg/g) and for nonsmoking research workers in Boston (50±24 pg/g) with reported little or no ETS exposure.[7] However, 3-ABP haemoglobin levels in the latter group (1.3±1.5 pg/g) are comparable with the levels found in pregnant nonsmoking women from Homburg (1.4±0.1 pg/g). The fact that 5 of 78 self-reported nonsmoking women had cotinine levels indicating recent active smoking underlines the importance of controlling for self-reported smoking status by use of a reliable biomarker. Interestingly, four of these five miss-classified nonsmokers claimed to be highly exposed to ETS.

Both miss-classification of self-reported smoking status and confounding effects on haemoglobin adduct levels due to exposure sources other than ETS may explain the high inter-individual variations and lack of correlation with ETS exposure. While no additional sources are known for HPB adducts derived from TSNA,[6] nitroaromatics may contribute to the aromatic amine adduct levels.[11]

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